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### **► To cite this version:**

Miao Feng, Ziane Elaïb, Delphine Borgel, Cécile Denis, Frédéric Adam, et al.. NAADP/SERCA3-dependent  $\text{Ca}^{2+}$  stores pathway specifically controls early autocrine ADP secretion potentiating platelet activation.. *Circulation Research*, 2020, 127 (7), pp.e166-e183. 10.1161/CIRCRESAHA.119.316090 . inserm-02948394

**HAL Id: inserm-02948394**

**<https://inserm.hal.science/inserm-02948394>**

Submitted on 24 Sep 2020

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**NAADP/SERCA3-dependent  $\text{Ca}^{2+}$  stores pathway specifically controls early autocrine ADP secretion potentiating platelet activation.**

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**Abstract:** 265 words; **Total word count:** 7973 words; 9 figures: 57 **references**

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**Short title:** NAADP and SERCA3-dependent activation pathway.

**Subject codes:** Cell Signaling/Signal Transduction, Platelets

## **Abstract**

### **Rationale:**

Ca<sup>2+</sup> signaling is a key and ubiquitous actor of cell organization and its modulation controls many cellular responses. Sarco-endoplasmic reticulum Ca<sup>2+</sup>-ATPases (SERCAs) pump Ca<sup>2+</sup> into internal stores that play a major role in the cytosolic Ca<sup>2+</sup> concentration rise upon cell activation. Platelets exhibit 2 types of SERCAs, SERCA2b and SERCA3, which may exert specific roles, yet ill-defined. We have recently shown that Ca<sup>2+</sup> mobilization from SERCA3-dependent stores was required for full platelet activation in weak stimulation conditions.

### **Objective:**

To uncover the signaling mechanisms associated with Ca<sup>2+</sup> mobilization from SERCA3-dependent stores leading to ADP secretion.

### **Methods and Results:**

Using platelets from wild-type or SERCA3-deficient mice, we demonstrated that an early (within 5 to 10 seconds following stimulation) secretion of ADP specifically dependent on SERCA3 stored Ca<sup>2+</sup> is exclusively mobilized by nicotinic acid adenosine dinucleotide phosphate (NAADP): both Ca<sup>2+</sup> mobilization from SERCA3-dependent stores and primary ADP secretion are blocked by the NAADP receptor antagonist Ned-19, and reciprocally both are stimulated by permeant NAADP. In contrast, Ca<sup>2+</sup> mobilization from SERCA3-dependent stores and primary ADP secretion were unaffected by inhibition of the production of inositol-1,4,5-trisphosphate (IP3) by phospholipase-C, and accordingly were not stimulated by permeant IP3.

### **Conclusions:**

Upon activation a NAADP/SERCA3 Ca<sup>2+</sup> mobilization pathway initiates an early ADP secretion, potentiating platelet activation, and a secondary wave of ADP secretion driven by both an IP3/SERCA2b-dependent Ca<sup>2+</sup> stores pathway and the NAADP/SERCA3 pathway. This does not exclude that Ca<sup>2+</sup> mobilized from SERCA3 stores may also enhance platelet global reactivity to agonists. Because of its modulating effect on platelet activation, this NAADP-SERCA3 pathway may be a relevant target for anti-thrombotic therapy.

### **Keywords**

SERCA3, calcium, NAADP, platelet, secretion

1	<b>Non-standard Abbreviation and Acronyms</b>	
2		
3	ADP	adenosine 5'-diphosphate
4	cCa <sup>2+</sup>	Cytosolic Ca <sup>2+</sup>
5	ER	Endoplasmic Reticulum
6	FITC	Fluorescein isothiocyanate
7	Fura-2-NM	Fura-2-NearMembrane-AM probe
8	IP3	inositol-1,4,5-trisphosphate
9	NAADP	nicotinic acid adenosine dinucleotide phosphate
10	OAG	1-Oleoyl-2-acetyl-sn-glycerol
11	PLC	phospholipase C
12	PMA	phorbol 12-myristate 13-acetate
13	PMCA <sub>s</sub>	Plasma Membrane Ca <sup>2+</sup> ATPases
14	PRP	Platelet-rich plasma ()
15	PPACK	d-Phe-Pro-Arg chloromethylketone dihydrochloride
16	SERCA <sub>s</sub>	Sarco-endoplasmic reticulum Ca <sup>2+</sup> -ATPases
17	Serca3 <sup>-/-</sup>	SERCA3 knockout mice
18	Tg	thapsigargin
19	tBHQ	2,5-dinucleotide-(tert-butyl)-1,4-benzohydroquinone
20	TxA <sub>2</sub>	Thromboxane A <sub>2</sub>
21	WT	wild-type mice
22		

## Introduction

Cytosolic  $\text{Ca}^{2+}$  ( $\text{cCa}^{2+}$ ) is central in cell signaling. The regulatory mechanisms of  $\text{cCa}^{2+}$  concentration involve entry from the extra cellular medium through several channels including, TRPC, or Orai-1 coupled to signaling (SOCE, Store Operated  $\text{Ca}^{2+}$  Entry).<sup>1-3</sup> The other  $\text{cCa}^{2+}$  sources are mitochondria and  $\text{Ca}^{2+}$  intracellular stores.  $\text{cCa}^{2+}$  is down-regulated by Na/Ca exchangers and Plasma Membrane  $\text{Ca}^{2+}$  ATPases (PMCA) that export  $\text{Ca}^{2+}$  to the outside, by uniporters that recapture  $\text{Ca}^{2+}$  into mitochondria (above a high threshold)<sup>4</sup> and most importantly by Sarco-Endoplasmic Reticulum  $\text{Ca}^{2+}$  ATPases (SERCAs) that stock  $\text{Ca}^{2+}$  into intracellular stores.<sup>5</sup> Platelets have long been used as a model for studying the regulation of  $\text{cCa}^{2+}$ , because these anucleate cells recapitulate all regulatory and signaling  $\text{Ca}^{2+}$  pathways, and express SERCAs in quantitative amounts, including SERCA3. Among these pathways, mobilization of  $\text{Ca}^{2+}$ , from SERCA-dependent internal stores, is described as an early event upstream of all other signaling pathways.  $\text{Ca}^{2+}$  mobilization is highly relevant since involved in platelet activation and engagement of integrin  $\alpha_{\text{IIb}}\beta_3$  in aggregation.<sup>6</sup> Platelet SERCAs have been particularly well characterized. SERCAs are encoded by 3 genes, *ATP2A1*, *ATP2A2* and *ATP2A3*, which produce several alternate transcripts and protein isoforms: SERCA1a/b, SERCA2a-c, and SERCA3a-f. They are found in multiple tissues but platelets exhibit only SERCA2b and SERCA3 isoforms.<sup>7-10</sup> SERCAs maintain a  $\text{Ca}^{2+}$  concentration gradient between the cytosol (100 nmol/L) and the Endoplasmic Reticulum (ER) (1 mmol/L).<sup>11</sup> However, several questions remain to be answered, among which: does the expression of multi-SERCA isoforms correlate with corresponding distinct effects and  $\text{Ca}^{2+}$  signaling organization in a given cell, here platelets?

SERCA enzymes share similar structures with distinct intrinsic activities:  $\text{Ca}^{2+}$  affinity higher for SERCA2b than for SERCA3 ( $K_{1/2} \sim 0.27 \mu\text{mol/L}$  versus  $1 \mu\text{mol/L}$ ) but  $\text{Ca}^{2+}$  uptake lower (7 nmol/min/mg of protein versus 21 nmol/min/mg, respectively)<sup>12, 13</sup> allowing both SERCAs to maintain  $\text{cCa}^{2+}$  at low levels in the resting cells.

Pathologies and mouse models provide insight into SERCA2b and SERCA3 functions. Mutations in the human *ATP2A2* gene affecting SERCA2, lead to the Darier's syndrome in humans, a dermatological syndrome.<sup>14, 15</sup> SERCA2 and SERCA3 mutations are associated with some cancers<sup>16-18</sup> suggesting involvement in cell differentiation.<sup>19, 20</sup> SERCA3 human mutations also seem associated with type II diabetes.<sup>21</sup> Mouse SERCA2 knockouts are not viable at the homozygous state, but heterozygotes exhibit SERCA2a- (defect in heart contractility and relaxation)<sup>22</sup> and SERCA2b-type defects, evocative of the Darier's syndrome.<sup>23</sup> Mouse SERCA3 knockouts (*Serca3*<sup>-/-</sup>) exhibit no phenotypic alterations,<sup>24</sup> except for an altered gustatory nerve response.<sup>25</sup> *In vitro*, impaired relaxation of *Serca3*<sup>-/-</sup> aorta rings was reported, with defective relaxation of vascular smooth muscle cells, altered  $\text{Ca}^{2+}$  signaling and low NO production.<sup>24</sup> *In vitro* also, low insulin secretion and altered  $\text{Ca}^{2+}$  oscillations were reported.<sup>26-28</sup> Altogether these results point to a potential specific role for SERCA3 in  $\text{Ca}^{2+}$  signal modulation.

In platelets, among other differences, immuno-electron microscopy localization studies suggested distinct topologies for SERCAs: peripheral for SERCA3, and more central for SERCA2b.<sup>29, 30</sup> Functional differentiation based on pharmacological studies, suggested association of SERCA3 with acidic  $\text{Ca}^{2+}$  stores,<sup>31</sup> as well as with STIM1, a  $\text{Ca}^{2+}$  sensor of store operated  $\text{Ca}^{2+}$  entry (SOCE) participating in  $\text{Ca}^{2+}$  stores replenishing by SERCA3.<sup>32</sup> We have recently reported that  $\text{Ca}^{2+}$  mobilization from  $\text{Ca}^{2+}$  stores dependent on SERCA3 but not on SERCA2b is involved in a secretion pathway important for weak agonist platelet stimulation.<sup>33</sup> Here, we show that this secretion of both ADP and ATP is an early secretory pathway within seconds following activation, and controlled by NAADP mobilizing  $\text{Ca}^{2+}$  from SERCA3-dependent stores (heretofore designated as SERCA3 stores) and independently from inositol-3-phosphate (IP3), which in turn triggers the bulk of secretion following  $\text{Ca}^{2+}$  mobilization from SERCA2b- dependent  $\text{Ca}^{2+}$  stores (heretofore designated as SERCA2b stores).

## Methods

The authors declare that all supporting data are available within the article [and its online supplementary files], and available from the corresponding author on reasonable request.

Online Data Supplement contains detailed description of methods.

## Results

### SERCA3-dependent ADP secretion occurs early and precedes SERCA2b-dependent secretion

We recently showed that a secretion, specifically triggered by  $\text{Ca}^{2+}$  mobilization from SERCA3 stores, amplifies low agonist platelet stimulation by thrombin (through the PAR4 receptor in mouse platelets), or collagen.<sup>33</sup> This pathway most likely involved ADP secretion, since ADP (but not ATP) addition to thrombin rescued the defect in platelets from SERCA3 deficient (Serca3<sup>-/-</sup>) mice, while its suppression by apyrase scavenging reduced activation of WT platelets to the level observed in Serca3<sup>-/-</sup> platelets, unaffected by apyrase.<sup>33</sup> However, direct evidence for SERCA3-dependent ADP release was missing, since only ATP secretion was measured. Here, we directly assessed ADP secreted from platelets as well as its kinetic, comparatively to ATP. 3 minutes after thrombin stimulation (40 mU/mL), ADP was detected in the supernatant of Serca3<sup>-/-</sup> mouse platelets to roughly half the level of WT platelets (Figure 1A), a ratio comparable to ATP (Figure 1B). Importantly, when secretion kinetics were assessed by thrombin inhibition by PPACK at different time points (Figure 1C; Online Figure IIA), ADP secretion was detectable in WT platelets as early as 5 seconds after thrombin stimulation, while not detected at all in Serca3<sup>-/-</sup> platelets. At 10 seconds, ADP secretion augmented in WT platelets and started rising in Serca3<sup>-/-</sup> platelets. After 3 minutes, ADP secretion rose to its maximal level in WT platelets, reaching only half that level in Serca3<sup>-/-</sup> platelets. A similar kinetics was seen for ATP (Figure 1D; Online Figure IIB), showing co-secretion of both molecules. Finally, confirming functional involvement of SERCA3 in early secretion, platelet pretreatment for 5 minutes with tBHQ (10  $\mu\text{mol/L}$ ), a SERCA3-specific pharmacological inhibitor,<sup>33</sup> suppressed early secretion (as assessed by ATP, Figure 1D) at 5 seconds in WT platelets, mimicking Serca3<sup>-/-</sup> platelets, expectedly unaffected by SERCA3 inhibition. Both followed the same ATP secretion kinetics up to 180 seconds. We concluded that within the first 5 seconds following platelet stimulation by thrombin, ADP and ATP secretions were exclusively dependent on SERCA3-controlled signaling, while SERCA3-independent secretions set off after 10 seconds, likely dependent on  $\text{Ca}^{2+}$  from SERCA2b stores, both pathways adding up or the SERCA3-dependent pathway potentiating the SERCA2b-dependent pathway.

### Specific detection of $\text{Ca}^{2+}$ mobilization from SERCA3 stores by the intracellular plasma membrane-associated probe Fura-2-NearMembrane

Since early ADP secretion may be essentially dependent on SERCA3, we postulated that  $\text{Ca}^{2+}$  mobilization from SERCA3 stores was the consequence of platelet activation and was independent of ADP, secreted ADP being then involved in reinforcement of  $\text{Ca}^{2+}$  mobilization from SERCA2b stores. To compare both mobilizations, we searched for  $\text{Ca}^{2+}$ -probes that might be specific for one SERCA and not the other. Considering that SERCA3 may be localized near the plasma membrane and SERCA2b more central (<sup>29, 31</sup> and unpublished results), we compared  $\text{Ca}^{2+}$  measurements obtained with the Fura-2-AM and with the Fura-2-NearMembrane-AM probe (Fura-2-NM), a derivative of the FFP18 probe modified to localize to the inner leaflet of membranes and to selectively detect intracellular  $\text{Ca}^{2+}$  concentration proximal to membranes.<sup>34</sup> Washed platelets, pre-loaded with either probe, were sedimented onto poly-D-lysine-coated coverslips and cytosolic  $\text{Ca}^{2+}$  concentration was assessed by videomicroscopy as described in Methods. WT or Serca3<sup>-/-</sup> platelets loaded with Fura-2 exhibited  $\text{Ca}^{2+}$  mobilization (in presence of extracellular EGTA) upon thrombin challenge (Figure 2A), visually slightly less intense for Serca3<sup>-/-</sup> platelets. In contrast, using Fura-2-NM, thrombin triggered a positive, though moderate, fluorescence signal in WT platelets and no fluorescence increase in Serca3<sup>-/-</sup> platelets (Figure 2B). Image quantification confirmed a stronger signal with Fura-2 than with Fura-2-NM in WT platelets (Figure 2C and 2E and Figure 2I and 2K). Interestingly, Serca3<sup>-/-</sup> platelets under Fura-2 exhibited reduced  $\text{Ca}^{2+}$  mobilization compared to WT platelets (Figure 2A, 2C and 3A), confirming our earlier results using flow cytometry.<sup>33</sup> The absence of detection of  $\text{Ca}^{2+}$  mobilization in Serca3<sup>-/-</sup> platelets loaded with Fura-2-NM (Figure 2E and 2K), suggests that Fura-2-NM does not detect  $\text{Ca}^{2+}$  mobilization from SERCA2b stores (the only  $\text{Ca}^{2+}$  stores left in Serca3<sup>-/-</sup> platelets). Furthermore, SERCA3 pharmacological inhibition with tBHQ resulted in the well-documented  $\text{Ca}^{2+}$  emptying of SERCA3 stores in resting WT platelets yielding a moderate mobilization signal (corresponding in fact to a  $\text{Ca}^{2+}$  leak usually compensated for by constitutive SERCA3 activity, pumping cytosolic  $\text{Ca}^{2+}$  back into stores) whether visualized with Fura-2 or Fura-2-NM (Figure 2D and 2F). In contrast and as expected, tBHQ did not induce any  $\text{Ca}^{2+}$  leak in Serca3<sup>-/-</sup> platelets prior to thrombin stimulation (Figure 2D and 2F), thus confirming absence or undetectable non-selective inhibition of SERCA2b at the concentration used (10  $\mu\text{mol/L}$ ). Note that unsolicited activation or signaling

desensitization by tBHQ-induced  $\text{Ca}^{2+}$  leakage from SERCA3-dependent stores in WT platelets was avoided by the 3 to 5 minutes preincubation time, to allow stabilization of cytosolic  $\text{Ca}^{2+}$ . In Fura-2-loaded platelets, tBHQ pretreatment lowered  $\text{Ca}^{2+}$  mobilization in response to thrombin in WT platelets to the level of Serca3<sup>-/-</sup> platelets (due to  $\text{Ca}^{2+}$  mobilization from the sole SERCA2b stores left) (Figure 2D), while no mobilization in response to thrombin was detected by Fura-2-NM (Figure 2F). In addition, the  $\text{Ca}^{2+}$  response to thrombin after tBHQ pretreatment of WT and Serca3<sup>-/-</sup> platelets, as visualized by Fura-2, was consistent with SERCA2b store release. The fact that Fura-2-NM only allowed visualization of tBHQ-induced calcium leakage from SERCA3 stores in WT platelets but no further mobilization in response to thrombin or in Serca3<sup>-/-</sup> platelets, suggested that Fura-2-NM is specific for  $\text{Ca}^{2+}$  exit from SERCA3 stores. As expected, inhibition of SERCA2b (the only SERCA left in Serca3<sup>-/-</sup> platelets) by thapsigargin (200nmol/L, conditions specific for SERCA2b, not affecting SERCA3 function)<sup>33</sup> abolished thrombin-induced  $\text{Ca}^{2+}$  mobilization in Serca3<sup>-/-</sup> but not in WT platelets (because of the SERCA3 stores which remain intact after thapsigargin), as visualized by both Fura-2 and Fura-2-NM (Figure 2G and 2H, respectively). Altogether, these results 1) indicate that the Fura-2-NM probe can be used as a specific probe for  $\text{Ca}^{2+}$  mobilization from SERCA3 stores, and 2) confirm a peripheral localization for  $\text{Ca}^{2+}$  mobilized from SERCA3 stores, consistent with its possible role in early secretion.

### **$\text{Ca}^{2+}$ mobilization from SERCA3 stores is independent of ADP.**

The specific monitoring, with Fura-2-NM, of  $\text{Ca}^{2+}$  mobilization from SERCA3 stores being established, we next analyzed the relationship between  $\text{Ca}^{2+}$  mobilization from SERCA3 stores and ADP. Control experiments showing  $\text{Ca}^{2+}$  mobilization in response to thrombin as detected with Fura-2 and Fura-2-NM are displayed respectively in Figure 2I and 2K. When observed with Fura-2, ADP scavenging by apyrase diminishes the overall  $\text{Ca}^{2+}$  mobilization in WT platelets down to the level of Serca3<sup>-/-</sup> platelets (Figure 2J), confirming our previous results by flow cytometry on platelets in suspension.<sup>33</sup> In contrast,  $\text{Ca}^{2+}$  mobilization from SERCA3 stores detected by Fura-2-NM in WT platelets was not affected by apyrase (Figure 2L). Conversely, exogenous ADP added to thrombin, while stimulating  $\text{Ca}^{2+}$  mobilization in Serca3<sup>-/-</sup> platelets up to the same level than WT platelets under Fura-2 (Figure 2M), did not modify  $\text{Ca}^{2+}$  mobilization in WT nor in Serca3<sup>-/-</sup> platelets as visualized by Fura-2-NM (Figure 2N). These results show that  $\text{Ca}^{2+}$  mobilization from SERCA3 stores is independent of ADP, consistent with the primary  $\text{Ca}^{2+}$  mobilization from SERCA3 stores taking place upstream of the initial ADP release.

### **$\text{Ca}^{2+}$ mobilization from SERCA3 stores is independent of the second messenger inositol-1,4,5 tris-phosphate**

We next checked whether differential  $\text{Ca}^{2+}$  mobilization between SERCA3 and SERCA2b stores, and sensitivity to the  $\text{Ca}^{2+}$  fluorophore Fura-2-NM, may extend to regulation by distinct signaling pathways. Inositol 1,4,5-tris phosphate (IP3) is the prominent second messenger driving  $\text{Ca}^{2+}$  mobilization from ER,<sup>35</sup> the recognized SERCA2b storing organelle.<sup>12</sup> To determine whether IP3 mobilized SERCA2b and/or SERCA3 stores, we analyzed  $\text{Ca}^{2+}$  mobilization in response to thrombin of platelets pre-treated with U73122, a well-characterized inhibitor of phospholipase C (PLC), the main IP3 source.<sup>36</sup> After assessing dose-dependent effects on SERCA2b-dependent  $\text{Ca}^{2+}$  mobilization using Serca3<sup>-/-</sup> mice as controls (Online Figure IIIA through IIID), we found, as visualized with Fura-2, that U73122 (0.75  $\mu\text{mol/L}$ ) completely suppressed  $\text{Ca}^{2+}$  mobilization in response to thrombin in Serca3<sup>-/-</sup> platelets (Figure 3A and Online Figure IIIC, IVA), indicating that IP3 mobilized  $\text{Ca}^{2+}$  essentially from SERCA2b stores (the only functional stores in Serca3<sup>-/-</sup> platelets).  $\text{Ca}^{2+}$  mobilization in WT platelets was affected by U73122, but only partially, consistent with SERCA3 stores mobilization independent from IP3. The fact that 1 $\mu\text{mol/L}$  U73122 resulted in complete inhibition of  $\text{Ca}^{2+}$  mobilization in WT platelets (Online Figure IIID) suggested some off-target effect at high concentration (such as was shown for PKC).<sup>37</sup> Interestingly, when visualized with Fura-2-NM, U73122 pretreatment did not affect  $\text{Ca}^{2+}$  mobilization from SERCA3 stores in WT platelets, while in Serca3<sup>-/-</sup> platelets,  $\text{Ca}^{2+}$  mobilization remained undetected (Figure 3B and Online Figure IVD). In Fura-2 loaded WT platelets, while pretreatment with both U73122 and Tg reduced  $\text{Ca}^{2+}$  mobilization in response to thrombin, pretreatment with both U73122 and tBHQ completely abolished it, further suggesting that IP3 mobilized calcium from SERCA2b stores and not from SERCA3 stores (Online Figure IIIE and IIIF, respectively).

Interestingly, addition of exogenous ADP to thrombin in the presence of U73122, did not restore mobilization in both WT and Serca3<sup>-/-</sup> platelets when visualized by Fura-2 (Figure 3A and Online Figure IVE), and did not affect it when visualized by Fura-2-NM (Figure 3B and Online Figure IVF). Altogether, these results are consistent with the paradigm of a  $\text{Ca}^{2+}$  mobilization from SERCA3 stores being independent of IP3. They also suggest that IP3 acts downstream of early ADP secretion and that this early ADP potentiates the PLC pathway, since blocked by U73122. Confirming that IP3 is not involved in  $\text{Ca}^{2+}$  mobilization from SERCA3 stores, exogenously added IP3-AM induced a similar  $\text{Ca}^{2+}$  mobilization in WT and Serca3<sup>-/-</sup> platelets under Fura-2 monitoring (Figure 3C), not detected using Fura-2-NM (Figure 3D). IP3-AM-induced  $\text{Ca}^{2+}$  mobilization was abolished in Fura-2 loaded platelets after treatment with Tg (depleting SERCA2b stores) but not with tBHQ (depleting SERCA3 stores) (Figure 3E, 3F and

3G), confirming that SERCA3 stores are not mobilized by IP3. The slight decrease in Ca<sup>2+</sup> mobilization induced by IP3-AM after tBHQ pre-treatment compared to IP3-AM alone for both WT and Serca3<sup>-/-</sup> platelets may be due to a limited non-selective effect of tBHQ, on SERCA2b,<sup>38</sup> or to a slight interference of tBHQ with IP3-AM.

#### **Ca<sup>2+</sup> mobilization from SERCA3 stores is triggered by nicotinic acid adenosine dinucleotide-phosphate.**

Two other second messengers, Nicotinic Acid Adenosine Dinucleotide-Phosphate (NAADP) and cyclic Adenosine Dinucleotide-Phosphate ribose (cADPr), are also known to elicit Ca<sup>2+</sup> mobilization,<sup>39</sup> though the presence and the role of cADPr in platelets is controversial.<sup>40</sup> Interestingly, NAADP has been shown to act as second messenger specific for mobilization of Ca<sup>2+</sup> from acidic stores in a wide range of cell types.<sup>41</sup> SERCA3 has been found associated with acidic stores in platelets,<sup>42</sup> and based on pharmacological studies, NAADP has been suggested to be a second messenger for platelet SERCA3 stores.<sup>31</sup> Here, we used the Serca3<sup>-/-</sup> mouse model to confirm and extend these observations. In Figure 4A and Online Figure VA and VC, as visualized with Fura-2, pre-incubation with Ned-19 (0.5 μmol/L) for 3 minutes, a well-characterized specific antagonist of NAADP,<sup>43</sup> diminished global Ca<sup>2+</sup> mobilization in response to thrombin in WT platelets to the level of Serca3<sup>-/-</sup> platelets, themselves unaffected by Ned-19 pretreatment. Similar results were obtained with BAPTA-Oregon green-loaded platelets analyzed by flow cytometry (Online Figure VIIA and VIIB). Moreover, addition of exogenous ADP simultaneously to thrombin bypassed Ned-19 inhibition and restored Ca<sup>2+</sup> mobilization in both WT and Serca3<sup>-/-</sup> platelets as visualized by Fura-2 (Figure 4A and Online Figure VE). This restored Ca<sup>2+</sup> mobilization appeared to be completely dependent on PLC activity since it was totally suppressed in both WT and Serca3<sup>-/-</sup> platelets when U73122 was added to Ned-19 (Figure 4A and Online Figure VG and VH), consistent with ADP triggering IP3-dependent Ca<sup>2+</sup> mobilization from SERCA2b stores. Of note, when visualized with Fura-2-NM, addition of Ned-19 suppressed thrombin Ca<sup>2+</sup> mobilization from SERCA3 stores in WT platelets (Figure 4B and Online Figure VB and VD), and as expected, this effect was not reversed by exogenous ADP (Figure 4B and Online Figure VF). These results thus strongly suggest that NAADP is the main second messenger mobilizing Ca<sup>2+</sup> from SERCA3 stores.

Consistent with involvement of NAADP in the mobilization of SERCA3 stores, NAADP-AM, in absence of thrombin, while exhibiting mobilization in WT platelets, did not mobilize Ca<sup>2+</sup> in Serca3<sup>-/-</sup> platelets visualized with both Fura-2 and Fura-2-NM (Figure 4C and 4D, and Online Figure VIA through VIC). Accordingly, pre-incubation with Ned-19 prevented NAADP-AM mediated Ca<sup>2+</sup> mobilization (Figure 4E and 4F, and Online Figure VIB and VIC). Depleting SERCA3 stores with the SERCA3 inhibitor tBHQ also prevented subsequent Ca<sup>2+</sup> mobilization by NAADP-AM in WT platelets (Figure 4G and 4H, and Online Figure VIB and VIC), while the SERCA2b-specific inhibitor thapsigargin (Tg, 200 nmol/L) was without effect and did not block Ca<sup>2+</sup> mobilization by NAADP-AM in WT platelets (Online Figure IXA, IXB). Similar observations were obtained with BAPTA-Oregon green-loaded platelets analyzed by flow cytometry (Online Figure VIID through F). Additionally, NAADP-AM induced Ca<sup>2+</sup> mobilization in WT platelets was not increased by ADP addition which, as expected, mobilized Ca<sup>2+</sup> in Serca3<sup>-/-</sup> platelets (Online Figure VIIIA through D).

Altogether, these results are consistent with a Ca<sup>2+</sup> mobilization from SERCA3 stores being specifically dependent on the second messenger NAADP, but not IP3.

#### **SERCA3-dependent primary secretion is mediated by NAADP.**

We next assessed whether the NAADP pathway was involved in early secretion. In Figure 5A, NAADP pathway inhibition by Ned-19 reduced secretion after thrombin stimulation in WT platelets to the level of Serca3<sup>-/-</sup> platelets, unaffected throughout secretion kinetics (5 seconds, 10 seconds and 3 minutes). Consistent with absence of effect on SERCA3 store mobilization, U73122 did not inhibit early secretion at 5 seconds in WT platelets, while blocking further secretion at later time points (Figure 5A), confirming that the primary secretion in WT platelets is independent of IP3. Likewise, addition of exogenous ADP to thrombin bypassed Ned-19 inhibition, restoring full secretion in both WT and Serca3<sup>-/-</sup> platelets (Figure 5B). These results confirm NAADP as second messenger specific for Ca<sup>2+</sup> mobilization from SERCA3 stores leading to early secretion. Interestingly, NAADP-AM, by itself, was unable to trigger secretion (Figure 5C), most likely because secretion requires not only Ca<sup>2+</sup> but also PKCs activation by diacylglycerol from PLC or/and PLD-mediated phospholipids break down.<sup>44</sup> We tested this possibility by addition of the PKC activator PMA (phorbol-myristyl-acetate, 10 nmol/L) (Figure 5C), after determination of the optimal concentration (Online Figure Xa). NAADP-AM in the presence of PMA triggered secretion in WT, but not in Serca3<sup>-/-</sup> platelets. Interestingly the level of secretion reached was comparable to that of thrombin 40 mU/ml within 5 to 10 seconds of stimulation (Figure 5A). The other PKC activator, OAG (1-Oleoyl-2-acetyl-*sn*-glycerol)<sup>45</sup> yielded similar results (Online Figure XB). IP3-AM in the presence of PMA induced secretion to comparable levels in Serca3<sup>-/-</sup> platelets and WT platelets, consistent with SERCA2b-dependent secretion (Figure 5D). Moreover, NAADP-AM in combination with PMA and IP3 amplified secretion of WT platelets (comparable to thrombin), but, as expected, not of Serca3<sup>-/-</sup> platelets (Figure 5D). Of note, no secretion was observed in presence of ADP alone



and the level of secretion induced in presence of PMA with 10  $\mu\text{mol/L}$  ADP was similar in WT and Serca3<sup>-/-</sup> platelets (not shown). In WT platelets, ADP+PMA-induced secretion was not significantly different from that obtained with NAADP and PMA, and was markedly lower than the secretion obtained with thrombin in WT platelets (Online Figure VIII E). Finally, we wished to verify the role of the NAADP pathway in platelet aggregation induced by low concentration of thrombin (40 mU/mL). Ned-19 demonstrated a partial inhibitory effect on aggregation of WT and not of Serca3<sup>-/-</sup> platelets (Figure 6A and 6B). Aggregation of both WT and Serca3<sup>-/-</sup> platelets in the presence of Ned-19 was completely restored by exogenous ADP (Figure 6C), consistent with the contention that ADP bypasses the SERCA3/NAADP-dependent pathway. U73122 inhibited aggregation to low thrombin concentration, partially for WT platelets, and completely for Serca3<sup>-/-</sup> platelets (Figure 6D). Moreover, Ned-19 exhibited an inhibitory effect additive to U73122 on WT platelets (Figure 6E), consistent with involvement of both the NAADP-SERCA3 and the IP3-SERCA2b pathways in aggregation.

### **Thromboxane A<sub>2</sub> is involved in the potentiation of thrombin signaling by the Serca3/NAADP pathway**

Thromboxane A<sub>2</sub> (TxA<sub>2</sub>) is an important autocrine agonist in the amplification step of platelet activation.<sup>46</sup> To determine whether it was involved in the SERCA3/NAADP pathway, we have assessed the response to thrombin of indomethacin-pretreated (5  $\mu\text{mol/L}$ , the most efficient concentration tested) platelets for 10 minutes. Indomethacin partially inhibited Ca<sup>2+</sup> mobilization in WT, but not in Serca3<sup>-/-</sup> platelets (Figure 7A through C). The inhibitory effect of indomethacin on Ca<sup>2+</sup> mobilization was not affected by Ned-19, nor rescued by ADP (Figure 7C). Interestingly indomethacin did not affect early secretion at 5 or 10 seconds of WT platelets, and only partially (about 25%) at 180 seconds (Figure 7D and 7E) in WT platelets. However, it completely inhibited secretion of Serca3<sup>-/-</sup> platelets at 180 seconds (Figure 7E), consistent with the known dependence of IP3 (and the Serca2b pathway) on TxA<sub>2</sub>. Accordingly, indomethacin prevented restoration of secretion by ADP of Serca3<sup>-/-</sup> platelets. These results argue against a role for TxA<sub>2</sub> in the SERCA3/NAADP pathway itself. Since it does not affect mobilization nor secretion in Serca3<sup>-/-</sup> platelets, this indicates also that TxA<sub>2</sub> does not play a role in Serca2b-dependent Ca<sup>2+</sup> store mobilization or secretion either. The partial inhibition of Ca<sup>2+</sup> mobilization and secretion in WT platelets is thus best explained by a role for TxA<sub>2</sub> at the level of the potentiation of thrombin signaling by ADP secreted by the SERCA3/NAADP pathway. Similar results were also observed in human platelets (Figure 8).

### **Human platelets exhibit also a SERCA3/NAADP pathway controlling an early ADP secretion potentiating activation.**

Because we previously demonstrated that human platelets also exhibited a SERCA3-dependent Ca<sup>2+</sup> mobilization controlling secretion,<sup>35</sup> we verified that, like for mouse platelets, this pathway was dependent on NAADP. Ca<sup>2+</sup> mobilization in response to thrombin was strongly decreased when human platelets were pretreated with either tBHQ or Ned-19 (Figure 8A and 8B). As expected, inhibition of thromboxane A<sub>2</sub> production with indomethacin, also strongly decreased Ca<sup>2+</sup> mobilization in response to thrombin, but ADP addition to thrombin did not restore Ca<sup>2+</sup> mobilization, contrasting with Ned-19 (Figure 8C). Furthermore, as expected, tBHQ and Ned-19 pretreatment blocked early ATP secretion measured at 5 seconds, but secretion induced by thrombin in indomethacin-pretreated platelets was similar with control (Figure 8D through F). Finally, at 180 seconds, while both Ned-19 and indomethacin pretreatment resulted in a significant decrease in secretion (Figure 8G and 8H), like in mouse platelets, only Ned-19 inhibition was by-passed in presence of extracellular ADP, suggesting that both human and mouse platelets share a SERCA3/NAADP pathway triggering platelet activation upstream of the TxA<sub>2</sub> pathway.

Altogether these results are consistent with two distinct platelet Ca<sup>2+</sup> stores controlling secretion differentially: the first Ca<sup>2+</sup> store, sensitive to tBHQ and Ned-19, is dependent on SERCA3, independent from ADP and rapidly mobilized by NAADP to elicit a quick initial ADP secretion; the second, sensitive to thapsigargin and U73122, dependent on SERCA2b is dependent in part on ADP secreted by the SERCA/NAADP pathway and mobilized by IP3, and triggers together with SERCA3 stores, the bulk of secretion (Online Figure XI).

### **Discussion**

We have recently reported that a specific secretion triggered by Ca<sup>2+</sup> mobilization of SERCA3 stores, was necessary for complete platelet activation in conditions of low concentration of agonists.<sup>33</sup> Here, we provide evidence that SERCA3-dependent Ca<sup>2+</sup> stores drive early phase secretion, though they are also involved throughout secretion. Furthermore, we demonstrate that both Ca<sup>2+</sup> stores are strictly mobilized by distinct second messengers: NAADP for SERCA3 stores, and IP3 for SERCA2b stores. Differential mobilization of SERCA3 and SERCA2b Ca<sup>2+</sup> has already been suggested by earlier studies,<sup>31, 39, 47</sup> but these were based only on pharmacological evidence, and

therefore lacked more direct proofs. The present study definitively establishes that SERCA3 and SERCA2b stores are differentially regulated, and more importantly link these mobilizations to distinct pathways of ADP secretion. We demonstrate a functional connection between  $\text{Ca}^{2+}$  mobilization from SERCA3 stores and rapid initial ADP secretion, that exhibits an autocrine function particularly relevant in conditions of platelet activation with low agonist concentrations. This pathway is triggered by NAADP, while the other pathway, taking place secondarily, is dependent on SERCA2b stores mobilized exclusively by IP3 (Online Figure XI).

The topological difference between SERCAs, SERCA3 being located at the periphery of the platelet membrane <sup>29, 30</sup> (and unpublished) where it participates in the regulation of the Store Operated Calcium Entry (SOCE) through the plasma membrane, <sup>32</sup> is in agreement with the fact that  $\text{Ca}^{2+}$  mobilization visualized with the Fura-2-NM-AM probe, designed to associate with the inner leaflet of the membrane, <sup>34, 48</sup> originated only from SERCA3 stores, but not from SERCA2b stores. This suggests that SERCA3 stores mobilization occurs preferentially near the plasma membrane, consistent with early secretion.

Of note, our observation shows that in addition to participating to early secretion, SERCA3 stores contribute to secretion throughout, since late secretion (3 min) is also defective in *Serca3*<sup>-/-</sup> platelets upon thrombin stimulation (Figure 1). Interestingly defective SERCA3-dependent secretion is not due to a global defect in cytosolic  $\text{Ca}^{2+}$  rise, since high thrombin concentration (0.1 mU/mL) which restores normal  $\text{Ca}^{2+}$  levels in *Serca3*<sup>-/-</sup> platelets, does not rescue defective secretion (data not shown, and ref 33, Figure 4). This thus argues against a simple control of cytosolic  $\text{Ca}^{2+}$  levels by SERCA3 stores, together with SERCA2b stores, in the regulation of secretion, and rather, points to a more specific role, such as control of peripheral  $\text{Ca}^{2+}$ . However, we do not exclude a role for SERCA3 in enhancing platelet reactivity to agonists, acting together with SERCA2b-dependent  $\text{Ca}^{2+}$  stores.

Our recording systems could not discriminate between SERCA3/NAADP and SERCA2b/IP3-dependent  $\text{Ca}^{2+}$  mobilization kinetics. It is possible that both mobilizations initiated within the same time frame. But our findings show that the PLC/IP3 pathway is amplified following  $\text{Ca}^{2+}$  mobilization from SERCA3 stores and ADP secretion. This means that SERCA3 stores might be mobilized first and suggests the existence of an NAADP store or the rapid generation of NAADP prior to IP3 production. A study in platelets on the role of CD38, a membrane enzyme that catalyzes the exchange of the nicotinamide group of NADP<sup>+</sup> with the nicotinic acid to synthesize NAADP, <sup>49</sup> suggested that CD38 products were involved in platelet functions. Others observed a decreased  $\text{Ca}^{2+}$  signal in a CD38<sup>-/-</sup> mouse model, or upon inhibition of NAADP-sensitive stores. <sup>39</sup> These authors also suggested that NAADP synthesis was secondary to IP3 synthesis, since NAADP production was reduced (not blocked) by the PLC inhibitor U73122. <sup>39</sup> Our results are clearly in contradiction with this observation since in our hands, NAADP-dependent mobilization takes place within the 5 first seconds of activation and is not sensitive to PLC inhibition by U73122. Different experimental conditions may explain these opposite results: these authors used high concentrations of thrombin, 500 mU/mL, more than 10 times higher than 40 mU/mL in our study. Also, platelets were subjected to aggregation which adds up activation signals as well as signaling downstream integrin  $\alpha_{\text{IIb}}\beta_3$ , while we focused on initial activation and used unstirred conditions to prevent  $\alpha_{\text{IIb}}\beta_3$  engagement and subsequent secretion.

One of our most relevant findings is that early ADP secretion appeared detectable within seconds of agonist stimulation of platelets and in a manner fully dependent on the SERCA3-NAADP pathway, while a secondary ADP secretion pathway follows, dependent on SERCA2b and IP3. The delay between SERCA3/NAADP-dependent and SERCA2b/IP3-dependent secretions could be due to differential signaling and/or secretory machinery: for example, SERCA3 stores which are peripheral will mobilize peripheral granules, thus inducing a quick exit of ADP, while SERCA2b stores, more central, will mobilize granules further away from the membrane and their exocytosis will take longer. The major consequence is that both pathways regulate secretion sequentially, at least initially. This temporal organization, with NAADP-dependent  $\text{Ca}^{2+}$  mobilization ahead of other mobilization pathways has already been observed in a number of other cell types, including sea urchin sperm, <sup>50</sup> pancreatic cells, <sup>51</sup> or T-lymphocytes. <sup>52, 53</sup> Though dense granule secretion (or equivalent, such as lysosomes) has not been documented in these cell types and correlated with  $\text{Ca}^{2+}$  mobilization differential pathways, our observation may be a general mechanism by which an NAADP-SERCA3  $\text{Ca}^{2+}$  mobilization pathway regulates an early secretion of functional relevance to trigger full activation of cells. The fact that  $\text{Ca}^{2+}$  mobilization from SERCA3 stores whether induced directly by NAADP-AM or by thrombin in presence of apyrase did not trigger a secondary  $\text{Ca}^{2+}$  mobilization suggests that, at least, in platelets, NAADP-dependent  $\text{Ca}^{2+}$  mobilization leads to secondary  $\text{Ca}^{2+}$  mobilization (from SERCA2b stores) not directly via Calcium Induced Calcium Release (CICR) but indirectly via ADP release and signaling. Furthermore, we observed that direct mobilization of  $\text{Ca}^{2+}$  using NAADP-AM was unable to induce primary secretion nor platelet aggregation, both requiring an additional stimulus. Only when NAADP-AM was associated to PMA or OAG-AM, both PKC activators, we observed an ADP secretion of the same level than that observed at 5 seconds of thrombin stimulation. This result suggests that  $\text{Ca}^{2+}$  mobilized from SERCA3 stores and diacyl-glycerol (DAG, from phospholipids breakdown by phospholipases) are needed to induce initial ADP secretion. It is possible that in physiological conditions most of the DAG is produced by PLC. However an

1 alternative possibility is that U73122 did not block initial ADP secretion because of another source of DAG,  
2 U73122-insensitive: potential candidates are phosphatidic acid phosphatases,<sup>54</sup> phospholipid transferases,  
3 monoglycerol acyl transferases, triacylglycerol lipases.<sup>55</sup> This possibility is currently under investigation.  
4 One issue raised by this study is related to the nature of the first wave of ADP (and ATP) release upon  
5 NAADP/SERCA3  $\text{Ca}^{2+}$  store mobilization: since only a fraction of the total content of dense granules is released,  
6 this brings up the possibility of 2 separate dense granule populations, each associated with one type of SERCA  $\text{Ca}^{2+}$   
7 stores signaling pathway: according to this hypothesis, one subtype of dense granules would be physically and/or  
8 functionally associated with SERCA3 stores, and would be the only granules able to release their content upon  $\text{Ca}^{2+}$   
9 mobilization from SERCA3 stores. The remaining granules would then enter the secretory pathway upon  $\text{Ca}^{2+}$   
10 mobilization from the SERCA2b stores. Alternatively, the dense granule population would be homogeneous but  
11 granules close to SERCA3 stores (possibly near the plasma membrane) would secrete their content upon  $\text{Ca}^{2+}$   
12 mobilization from these stores. Distinguishing between these two possibilities will require further investigations.  
13 Finally, we recently observed in platelets from patients with severe obesity, a pathological condition associated  
14 with increased circulating pro-thrombotic factors, an acquired decrease in SERCA3 expression associated with  
15 lower ability of platelets to get activated.<sup>56</sup> This observation suggests that targeting the NAADP/SERCA3 signaling  
16 pathway could be a way to limit spontaneous platelet activation, representing a potential future anti-thrombotic drug  
17 therapy.

## Acknowledgements

We thank Christelle Repérant for her technical help with platelet preparation and flow cytometry. We also thank UMS44 for technically and facility supports.

## Sources of funding

This work was supported in part by Fondation de France (engt:00086505), INSERM and Université Paris-Saclay, fellowships from the MENSUR and the Société Française d'Hématologie (SFH) to Z.E and a fellowship from the China Scholarship Council to M.F. At the time of the study Z.E. was a PhD candidate at Université Paris-Sud. M.F. is a PhD student at Université Paris-Sud.

## Disclosure of conflicts-of-interest

The authors declare no conflict-of-interest.

## Supplemental materials

Online Materials and method details

Online Figures I-XI

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## Figure legends

### Figure 1. SERCA3-dependent ADP secretion occurs early and precedes SERCA2b-dependent secretion.

WT (open squares) and Serca3<sup>-/-</sup> (gray circles) washed platelets in suspension ( $9 \times 10^7$  in 300  $\mu$ L) were stimulated with thrombin (40 mU/mL) for 3 min and released ADP (A) or ATP (B) in the supernatants (10  $\mu$ L) were assessed (see Methods). Data are expressed as dot plots and means  $\pm$  SEM using 2-way ANOVA, followed by Tukey's post-test,  $n=3$ . The kinetics of secretion of ADP (C) and ATP (D) were assessed in the same conditions, but thrombin stimulation was stopped at indicated time points by addition of the thrombin inhibitor D-Phe-Pro-Arg-chloromethylketone (PPACK, 100  $\mu$ M) and immediate chilling of platelets on ice and pelleting at 4°C. To demonstrate functional involvement of SERCA3 during secretion, tBHQ (10  $\mu$ M) was preincubated with platelets for 5 min prior to thrombin stimulation (40 mU/mL) and ATP secretion measured in the supernatant of WT (open triangles) or Serca3<sup>-/-</sup> (gray triangles) platelets at 0, 5, 10 and 180 seconds. Data are expressed as dot plots and means  $\pm$  SEM, 2-way ANOVA, followed by Tukey's multiple comparison test,  $n=3$  (C) and dot plots and means  $\pm$  SEM, 3-way ANOVA, followed by Tukey's multiple comparison test,  $n=4$  (D).

### Figure 2. Ca<sup>2+</sup> mobilization from SERCA3 stores is specifically detected by the intracellular plasma membrane-associated probe Fura-2-NearMembrane and is independent of ADP.

Washed platelets immobilized on glass coverslips coated with poly-D-lysine, were loaded with Fura-2-AM (Fura-2, left panel & tracings) or Fura-2-NearMembrane-AM (Fura-2-NM, right panel & tracings), and activated with thrombin (50 mU/mL). Fluorescence intensity images were recorded at 340 nm and 380 nm using videomicroscopy. As described in methods, cCa<sup>2+</sup> concentration ([Ca<sup>2+</sup>]) in WT and Serca3<sup>-/-</sup> platelets was visualized by pseudocolor imaging generated from the ratio of fluorescence intensities before and after thrombin stimulation with Fura-2 (A) or Fura-2-NM (B). Scale bar = 5  $\mu$ m. The kinetics of [Ca<sup>2+</sup>] intensity were assessed in Arbitrary Units (AU) using the 340/380 nm excitation fluorescence ratio, the value 1 corresponding to the basal level. WT (black tracings), and Serca3<sup>-/-</sup> (gray tracings) platelets were stimulated with thrombin alone, and visualized with Fura-2 (C) or Fura-2-NM (E). To assess SERCA3 functional involvement, platelets labeled with Fura-2 (D, G) or Fura-2-NM (F, H) were incubated with the SERCA3 inhibitor tBHQ (10  $\mu$ M) (D, F), or the SERCA2b inhibitor thapsigargin (Tg, 200 nM) (G, H), and challenged with thrombin (50 mU/mL). Recordings shown are representative of 3 independent experiments.

To assess the role of ADP, platelets were activated with thrombin (Thr) alone (50 mU/mL) (I, K, or in the presence of the nucleotide phosphate scavenger apyrase (APY, 10 U/mL) (J, L), or of ADP (10  $\mu$ M) (M, N). Data presented are representative of 3 independent experiments. Scale bar = 1 min.

### Figure 3. Ca<sup>2+</sup> mobilization from SERCA3 stores is independent of IP3.

Washed WT or Serca3<sup>-/-</sup> platelets preloaded with Fura-2 (A) or Fura-2-NM (B) were immobilized on poly-D-lysine glass coverslips and fluorescence intensity at 340 & 380nm was recorded using videomicroscopy. Platelets were preincubated for 1 minute with DMF (N, N-dimethylformamide) as control or with 0.75  $\mu$ M U73122 (in order to block IP3 production) before stimulation by thrombin (Thr) alone (50 mU/mL) or Thr in the presence of ADP (10  $\mu$ M). Ca<sup>2+</sup> mobilization visualized under Fura-2-AM (Fura-2) or Fura-2-NM (Fura-2-NM) was quantitated as area under curves (tracings shown in Supplemental Figures S4) for a time lapse of 2 min, relative to thrombin in WT platelets taken as 100%: open squares, WT and gray circles, Serca3<sup>-/-</sup>; absence (-) or presence (+) of U73122 or ADP are indicated below the graph. Data are presented as dot plots and means  $\pm$  SEM, using the 2-way ANOVA followed by Tukey's multiple comparison test (a)  $n=4$  (b)  $n=3$ . WT (black tracings) and Serca3<sup>-/-</sup> (gray tracings) platelets, preloaded with Fura-2 (C) or with Fura-2-NM (D), and immobilized on poly-D-lysine glass coverslips, were stimulated with IP3-AM (IP3) (10  $\mu$ M). The images taken before and after IP3-AM stimulation are presented in pseudocolors corresponding to the 340/380 ratio of fluorescence intensity (scale on the right) as visualized with Fura-2 (C) or with Fura-2-NM (D) and corresponding curves are presented below the images. Scale bar = 5  $\mu$ m. In (E) and (F) Fura-2 loaded platelets were pretreated with either Tg (200 nM) for 8 min or with tBHQ (10  $\mu$ M) for 5 min, respectively, before stimulation with IP3-AM (10  $\mu$ M). (G): Quantitation of Ca<sup>2+</sup> mobilization in WT or Serca3<sup>-/-</sup> platelets under Fura-2 was assessed by areas under curves for a time lapse of 2 min. WT (open squares) and Serca3<sup>-/-</sup> (gray circles); absence (-) or presence (+) of IP3, Tg ( $n=5$ ) or tBHQ ( $n=6$ ) are indicated below the graphs. Data are expressed as dot plots and means  $\pm$  SEM, using the 2-way ANOVA followed by Tukey's multiple comparison test.



**Figure 4.  $\text{Ca}^{2+}$  mobilization of SERCA3 stores is blocked by the NAADP antagonist Ned-19 and directly stimulated with NAADP.**

Washed platelets preloaded with Fura-2 (A, C, E, G) or Fura-2-NM (B, D, F, H) were immobilized on poly-D-lysine glass coverslips and fluorescence intensity at 340 & 380 nm was recorded using videomicroscopy. (A, B) Platelets were pre-incubated with either DMSO, U73122 (0.75  $\mu\text{M}$ ) or the NAADP receptor inhibitor Ned-19 (0.5  $\mu\text{M}$ ) and stimulated with thrombin (50 mU/mL) or thrombin and ADP (10  $\mu\text{M}$ ). Areas under curves (tracings shown in Online Figure V) for 2 min were quantitated and expressed as % of the thrombin response of WT platelets. WT platelets, open squares; Serca3<sup>-/-</sup> platelets, gray circles; absence (-) or presence (+) of thrombin (Thr), Ned-19, ADP or U73122 is indicated below the graphs. Data are presented as dot plots and means  $\pm$  SEM using the 2-way ANOVA followed by Tukey's multiple comparison test (A) n=4, (B) n=3.

Platelets were then challenged with NAADP-AM (NAADP) (1  $\mu\text{M}$ ) alone (C, D), or after preincubation with either Ned-19 (0.5  $\mu\text{M}$ ) (E, F), or tBHQ (10  $\mu\text{M}$ ) (G, H). [ $\text{Ca}^{2+}$ ] was calculated as indicated in Methods as relative fluorescent ratios to basal level. Scale bar = 1 min. Areas under curves for 2 min, visualized with Fura-2 or Fura-2-NM, are displayed in Online Figure VI. Data are representative of 3 to 4 experiments.

**Figure 5. Role of SERCA3, IP3 and NAADP in secretion.**

Washed WT (open squares) or Serca3<sup>-/-</sup> (gray circles) platelets were activated with thrombin (40 mU/mL), then the reaction was stopped by addition of PPACK (100  $\mu\text{M}$ ) at different time points (5, 10 or 180 sec), and platelet chilling on ice before centrifugation. ATP was assessed (in pmoles) in supernatants as described in Methods. (A) As indicated by (+), platelets were pretreated with Ned-19 (0.5  $\mu\text{M}$ ) or U73122 (0.75  $\mu\text{M}$ ). Data are presented as dot plots and means  $\pm$  SEM, n=4, using the 2-way ANOVA followed by Tukey's multiple comparison test. (B) Restoration of ATP secretion by ADP (10  $\mu\text{M}$ ) added to thrombin (Thr, 40 mU/mL) was assessed after 180 sec in the supernatant of WT (open squares) or Serca3<sup>-/-</sup> (gray circles) platelets pretreated (+) or not (-) with Ned-19. Data are presented as dot plots and means  $\pm$  SEM, n=3, using the 2-way ANOVA followed by Tukey's multiple comparison test. (C) Secretion induced by NAADP-AM (NAADP, 1  $\mu\text{M}$ ) for 180 sec was assessed in absence (-) or presence (+) of 10 nM PMA. Data are presented as dot plots and means  $\pm$  SEM, n=4, using the 2-way ANOVA followed by Bonferroni's multiple comparison test. (D) Comparative secretion at 180 sec as induced by NAADP (1  $\mu\text{M}$ ), PMA (10 nM) or IP3-AM (IP3, 10  $\mu\text{M}$ ) alone or in combination for 180 sec. Thrombin (Thr, 40 mU/mL, 180 sec) is used as positive control. (-) absence, (+) presence of agonist. Data are presented as dot plots and means  $\pm$  SEM, n=8 or n=3 (for combination of NAADP, IP3 and PMA), using the mixed-effects analysis (REML) followed by Tukey's multiple comparison test.

**Figure 6. Role of SERCA3, IP3 and NAADP in aggregation.**

Washed WT (black tracings) or Serca3<sup>-/-</sup> (gray tracings) platelets were subjected to aggregation in a Chronolog Lumiaggregometer (Chrono-Log Corporation, USA) after activation with thrombin (40 mU/mL) (A), in the presence of Ned-19 (0.5  $\mu\text{M}$ ) (B), Ned-19 and ADP (10  $\mu\text{M}$ ) (C), U73122 (0.75  $\mu\text{M}$ ) (D) or U73122 and Ned-19 (E). Arrows indicate the time point of agonist addition. Scale bar = 1 min. Aggregation recordings are representative of at least 3 independent experiments.

**Figure 7. The SERCA3/NAADP pathway is independent of thromboxane  $\text{A}_2$ .**

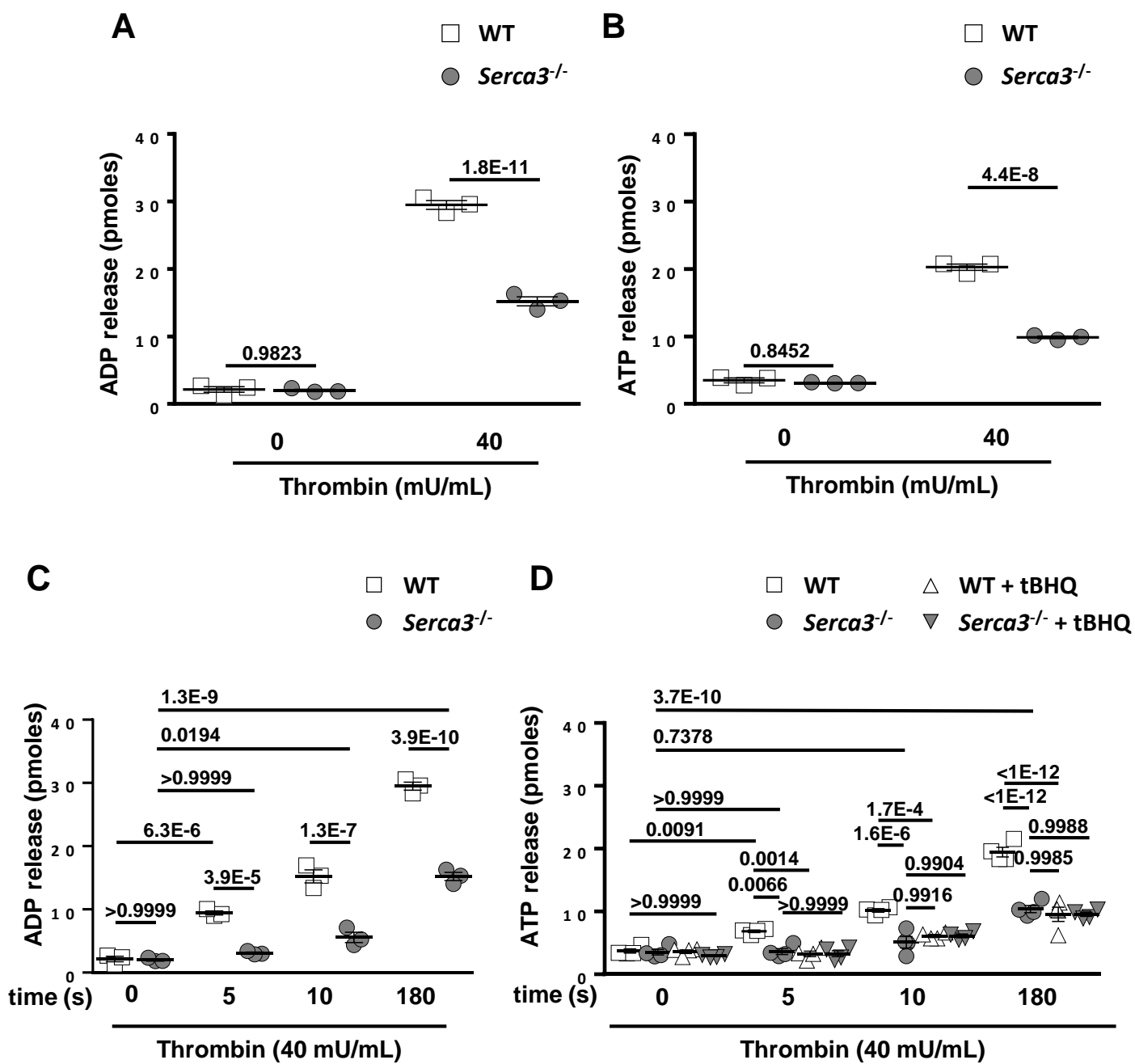
BAPTA-Oregon green loaded platelets from WT (black tracings) or Serca3<sup>-/-</sup> (gray tracings) animals, pretreated or not (A, C) with indomethacin (B, C) for 10 min alone (n=6) or in combination with Ned-19 (0.5  $\mu\text{M}$ ) (n=3) for 3 min (c) were analyzed for  $\text{Ca}^{2+}$  mobilization in response to thrombin 40mU/mL (Thr) using flow cytometry. Restoration of  $\text{Ca}^{2+}$  mobilization by ADP (10  $\mu\text{M}$ ) added to thrombin in indomethacin pretreated platelets was assessed (n=3). Areas under curves for 2 min were quantitated and expressed as % of the thrombin response of WT platelets taken as 100%. WT platelets, open squares; Serca3<sup>-/-</sup> platelets, gray circles; absence (-) or presence (+) of Ned-19, Indomethacin (Indo) or ADP is indicated below the graphs. Data are expressed as dot plots and means  $\pm$  SEM, using the mixed effects analysis (REML) followed by Bonferroni's multiple comparison test. (D) Washed WT (open squares) or Serca3<sup>-/-</sup> (gray circles) platelets, pretreated for 10 min with indomethacin (5  $\mu\text{M}$ ) (open triangles and gray triangles for WT and Serca3<sup>-/-</sup> platelets respectively) were activated with thrombin (40 mU/mL), then the reaction was stopped by addition of PPACK (100  $\mu\text{M}$ ) and platelet chilling and centrifuging at different time points of 0 sec, 5 sec, 10 sec or 180 sec, and ATP was assessed (in pmoles) in supernatants as described in Methods. Data are expressed as dot plots (n=6) and means  $\pm$  SEM, using the 3-way ANOVA followed by Tukey's multiple comparison test. (E) WT (open squares) or Serca3<sup>-/-</sup> (gray circles) platelets pretreated for 10 min with (+) or not (-) with indomethacin (5  $\mu\text{M}$ ) (n=8) and/or Ned-19 (0.5  $\mu\text{M}$ ) (n=3) for 10 and 3 min, respectively, prior activation with thrombin for 180 sec. Restoration of ATP secretion by ADP (10  $\mu\text{M}$ ) added (n=3) to thrombin (Thr,

40 mU/mL) in indomethacin pretreated platelets was assessed. Thrombin (Thr, 40 mU/mL, 180 sec) is used as positive control. Data are presented as dot plots and means  $\pm$  SEM, using the mixed-effects analysis (REML) followed by Bonferroni's multiple comparison test.

**Figure 8. Human platelets display early ADP secretion dependent on a SERCA3/NAADP pathway.** (A) For  $\text{Ca}^{2+}$  mobilization experiments, human platelets were loaded with BAPTA-Oregon green 488 and pretreated with 10  $\mu\text{M}$  tBQH for 5 min (gray tracing), 0.5  $\mu\text{M}$  Ned-19 for 3 min (dot tracing) or 5  $\mu\text{M}$  indomethacin for 10 min (thin black tracing) or solvent (thick black tracing) before flow cytometry analysis of  $\text{Ca}^{2+}$  mobilization in response to thrombin (Thr, 40 mU/mL). (B) Areas under curves for 2 min were quantitated and expressed as % of the thrombin response taken as 100% (black squares). The presence of tBHQ (open squares), Ned-19 (grey squares) or Indomethacin (Indo, black & white squares) is indicated below the graphs. Data are presented as dot plots with means  $\pm$  SEM, n = 4, 1-way ANOVA followed by Tukey's multiple comparison test. (C) human platelets pretreated with 0.5  $\mu\text{M}$  Ned-19 for 3 min (circles) or 5  $\mu\text{M}$  indomethacin for 10 min (triangles) were stimulated with thrombin (50 mU/mL) (open) or thrombin and ADP (10  $\mu\text{M}$ ) (grey). Areas under curves for 2 min were quantitated and expressed as % of the thrombin response in untreated platelets (black squares) taken as 100%. Data are representative of 3 experiments, and expressed as dot plots with means  $\pm$  SEM, 1-way ANOVA followed by Tukey's multiple comparison test.

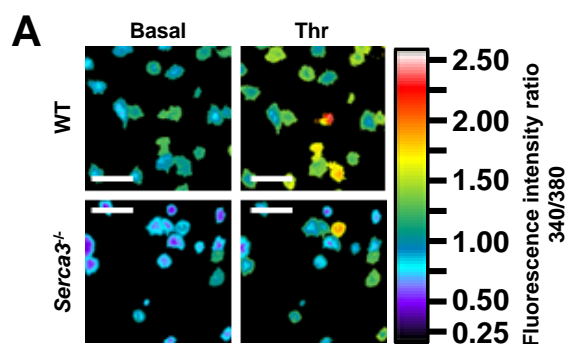
For secretion experiments, washed platelets were activated with thrombin (40 mU/mL), then the reaction was stopped by the PPACK/ice method at 5 sec (D), 10 sec (E) or 180 sec (F), and ATP was assessed (in pmoles) in supernatants as described in Methods. As indicated, platelets were pretreated with 10  $\mu\text{M}$  tBHQ for 5 min (open squares), 0.5  $\mu\text{M}$  Ned-19 for 3 min (grey squares) or 5  $\mu\text{M}$  indomethacin for 10 min (black & white squares) or solvent (black squares). Data are expressed as dot plots with means  $\pm$  SEM, n=3, 1-way ANOVA followed by Tukey's multiple comparison test. (G and H) Washed platelets were pretreated with 0.5  $\mu\text{M}$  Ned-19 for 3min or 5  $\mu\text{M}$  indomethacin for 10 min and were then activated with thrombin (50 mU/mL) (open squares) or thrombin and ADP (10  $\mu\text{M}$ ) (grey squares). Data are presented as dot plots with means  $\pm$  SEM, n=4, 2-way ANOVA followed by Tukey's multiple comparison test.

Figure 1

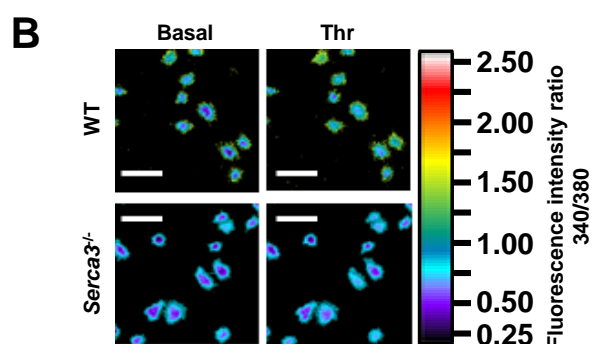


**Figure 2**

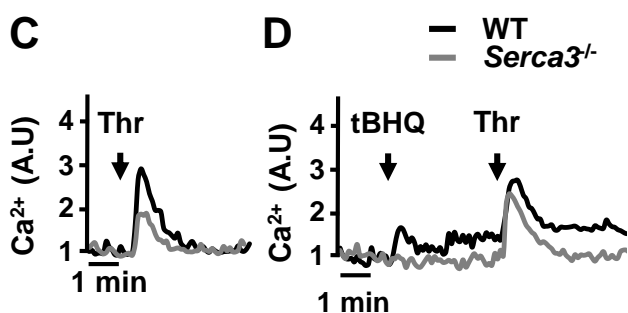
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*Fura-2-NM*



*Fura-2*



*Fura-2-NM*

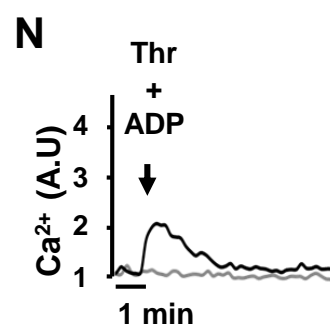
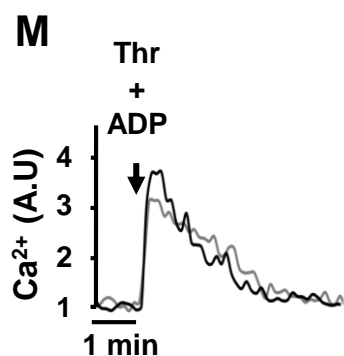
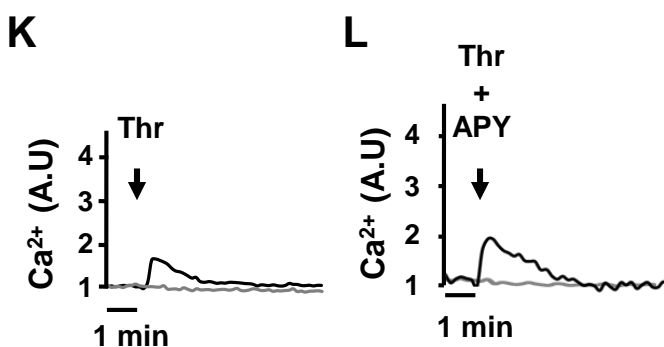
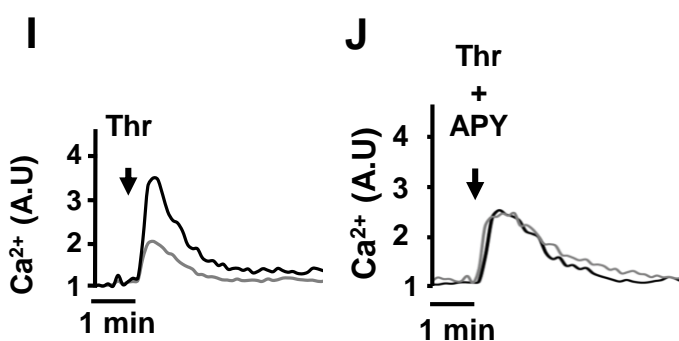
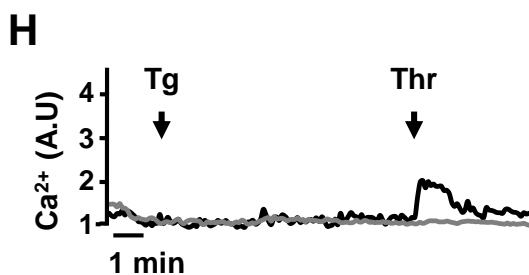
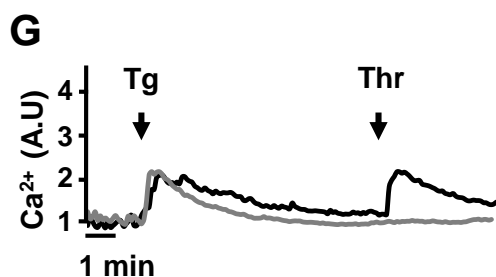
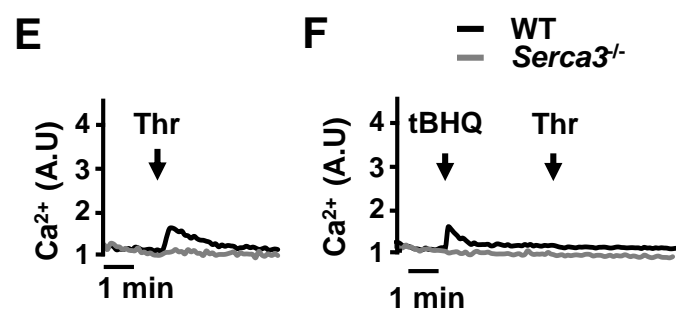


Figure 3

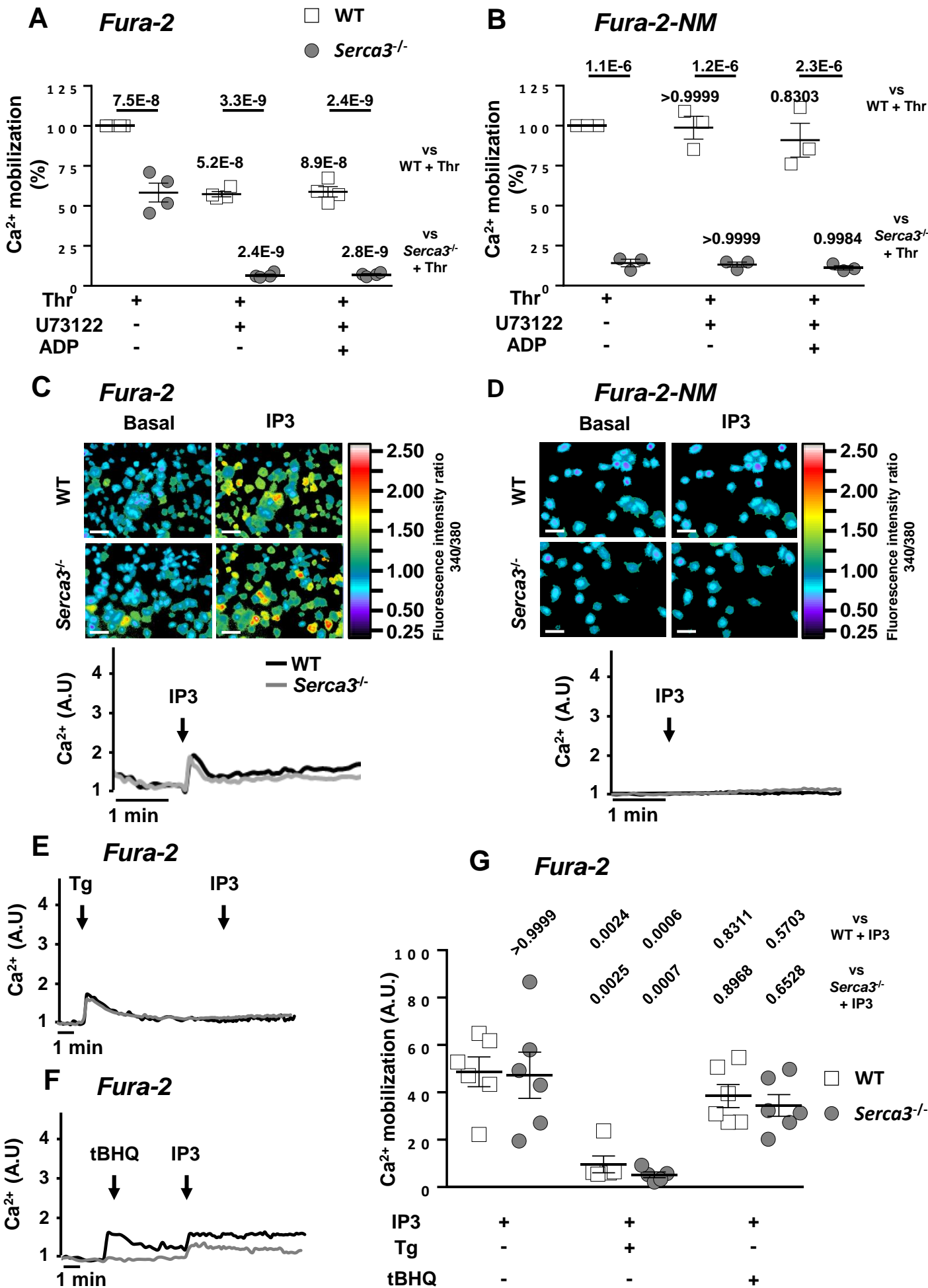


Figure 4

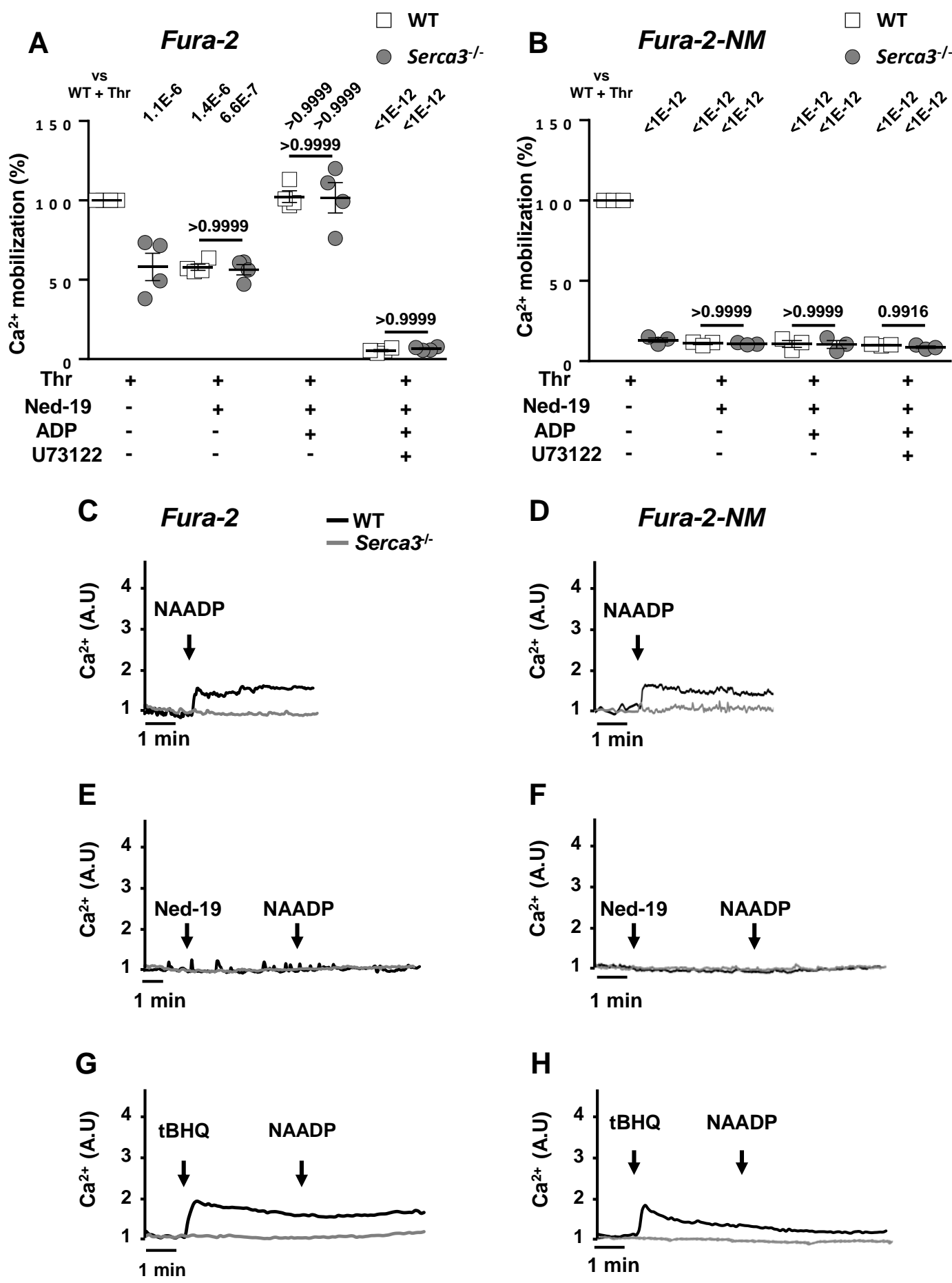


Figure 5

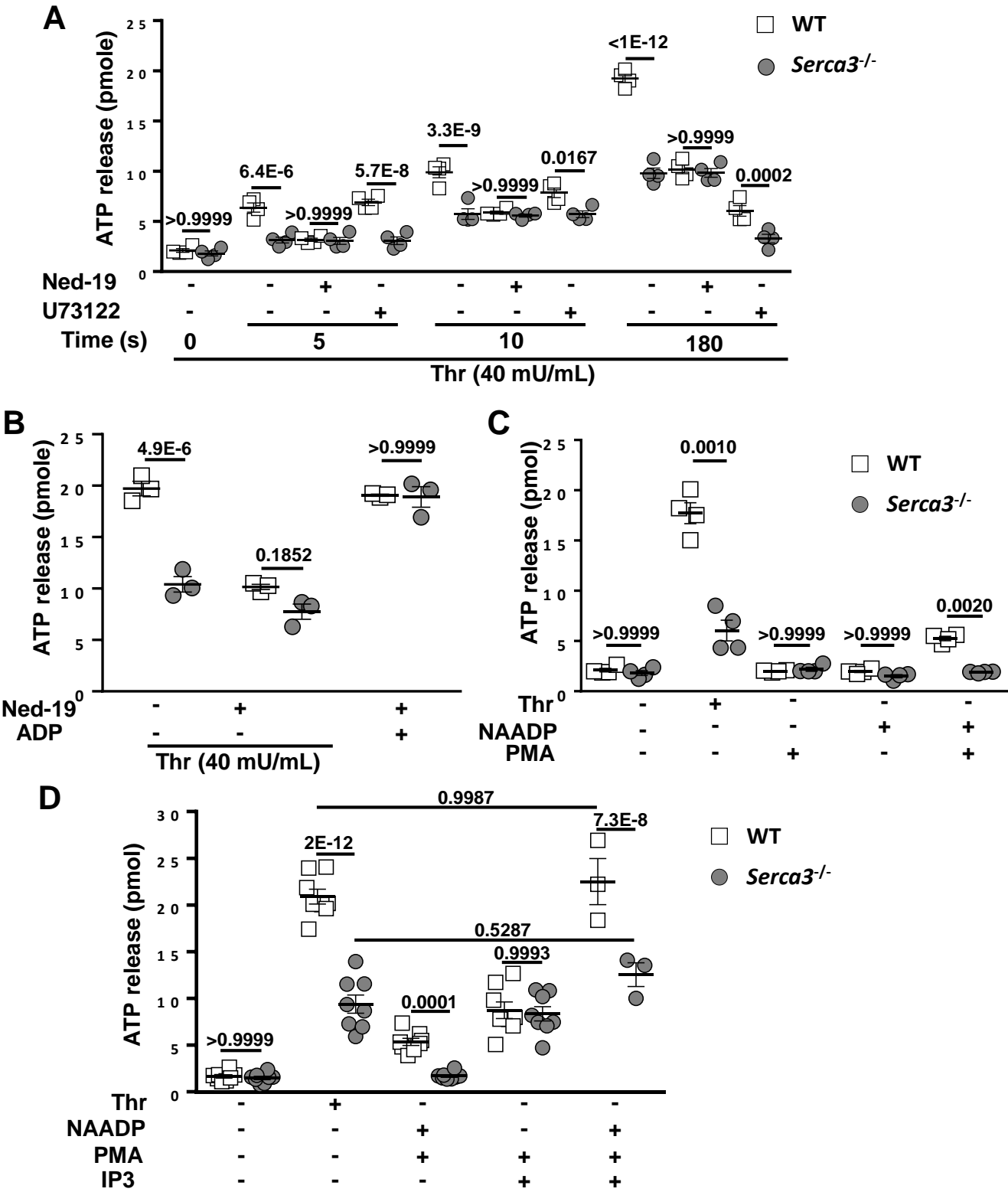


Figure 6

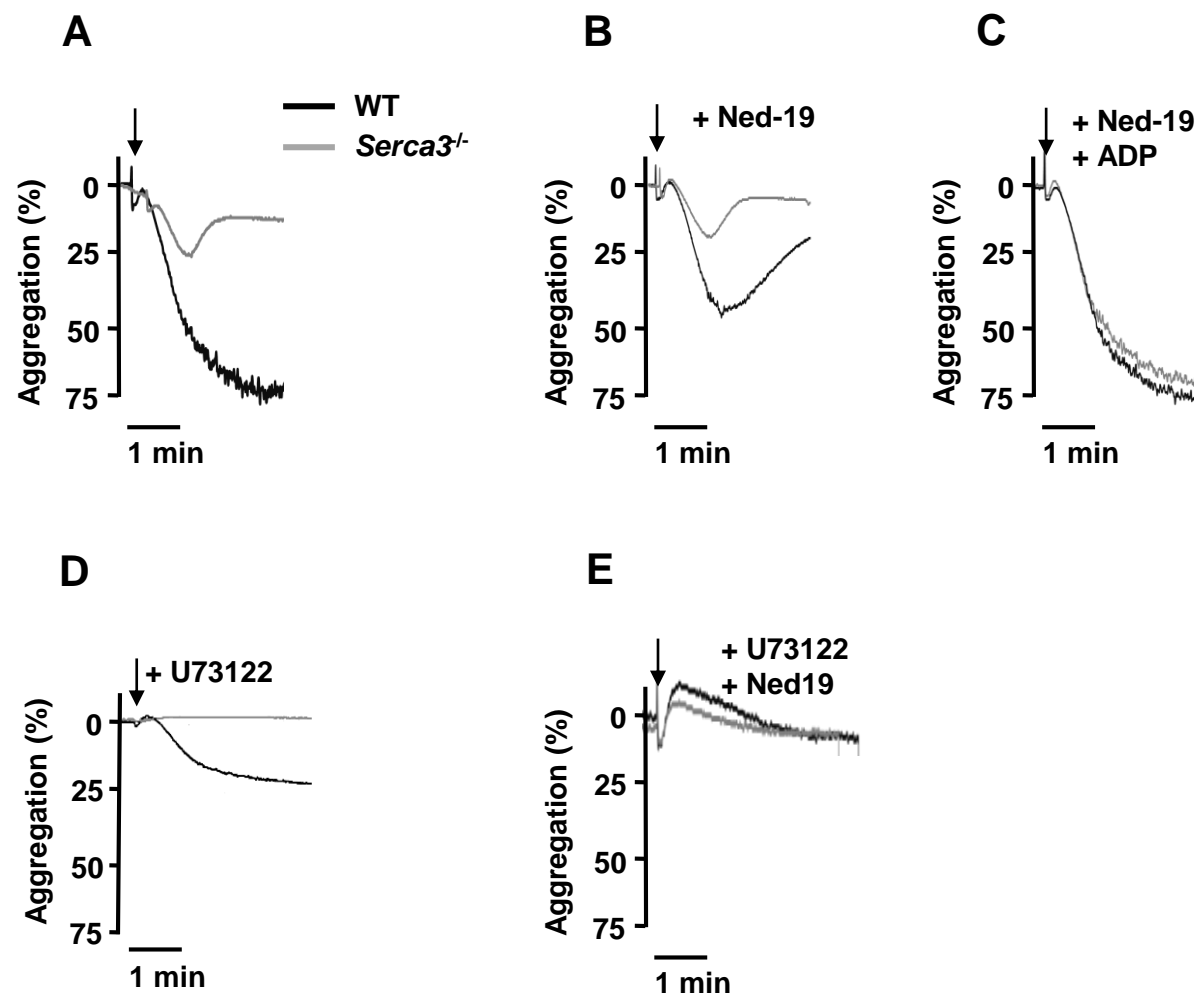




Figure 7

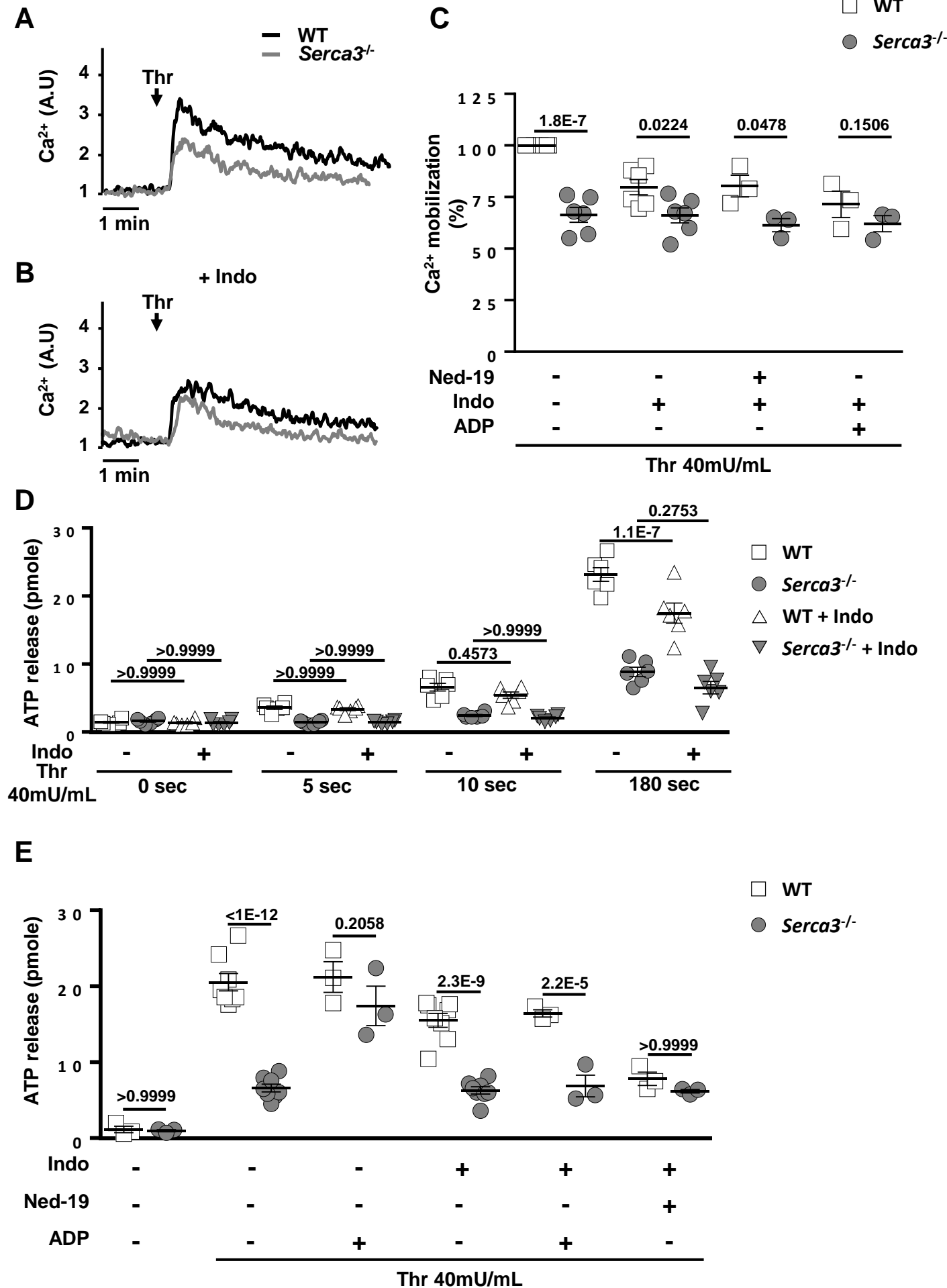


Figure 8

